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# Increased Immunoreactivity to Two Overlapping Peptides of Myelin Proteolipid Protein in Multiple Sclerosis

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## Summary

We tested the proliferative responses of peripheral blood mononuclear cells from 61 patients with multiple sclerosis, 56 healthy control subjects and 52 patients with other neurological diseases to seven synthetic peptides of myelin proteolipid protein (PLP) and 19 synthetic peptides of myelin basic protein (MBP). Increased proliferative responses to two overlapping PLP peptides, $PLP_{184-199}$ and $PLP_{190-209,}$ were found significantly more frequently in blood from patients with relapsing–remitting or secondary progressive multiple sclerosis (52.3%), but not from those with primary progressive multiple sclerosis (18.2%), than in that from healthy control subjects (8.9%) and patients with other neurological diseases (20.8%). Reactivity to these PLP peptides was most frequently seen in blood from patients with multiple sclerosis of 6–15 years duration and with moderate to severe disability (Kurtzke's Expanded Disability Status Scale > 4.0); the blood from 15 of 19 patients in this group reacted to one or both of the peptides. Both peptides could be recognized by short-term T-cell lines specific for whole PLP, and lines specific for one or other of the two overlapping peptides were able to recognize whole PLP, indicating that these peptides can be processed naturally from the intact molecule. This region of PLP is encephalitogenic in a number of strains of mice. Samples from multiple sclerosis patients did not react more frequently to any of the MBP peptides than those from healthy control subjects. The proportions of patients with other neurological diseases whose blood responded to the MBP peptides that most frequently elicited responses in blood from multiple sclerosis patients were significantly lower than the proportions of multiple sclerosis patients and healthy control subjects whose blood responded to these peptides.

**Keywords:** multiple sclerosis; proteolipid protein; immunoreactivity; myelin basic protein; auto-immunity

**Abbreviations:** ANOVA = analysis of variance; EAE = experimental autoimmune encephalomyelitis; EDSS = Kurtzke's Expanded Disability Status Scale; HLA = human leucocyte antigen; MBP = myelin basic protein; MHC = major histocompatibility complex; PBMC = peripheral blood mononuclear cells; PCR = polymerase chain reaction; PLP = myelin proteolipid protein; SI = stimulation index

## Introduction

Multiple sclerosis is a human demyelinating disorder that is likely to have an autoimmune basis (Pender, 1995). As the demyelinated lesions in multiple sclerosis are usually restricted to the CNS (Lumsden, 1970; Sobel, 1995), it is likely that the target antigens of autoaggressive cells in multiple sclerosis are expressed primarily or exclusively in the CNS.

CNS myelin proteins are possible targets. The most abundant proteins of CNS myelin are proteolipid protein (PLP) and myelin basic protein (MBP). PLP comprises more than 50% ofthe protein in CNS myelin. It is expressed in small amounts (< 0.5% of total protein) by Schwann cells in the PNS (peripheral nervous system), but it is not incorporated into PNS myelin (Puckett *et al.,* 1987). In experimental animals, immunization with PLP and adjuvants results in demyelinated lesions that are restricted to the CNS (Tuohy *et al.,* 1988; Greer *et al.,* 1992, 1996*a*; Chalk *et al.,* 1994*a*, *b),* as in multiple sclerosis. In contrast, MBP is found in significant quantities (20–30% of total protein) in both CNS and PNS myelin and, in experimental animals, immunization with MBP (or whole CNS tissue) and adjuvants or the passive transfer of MBP-specific lymphocytes results in demyelinated lesions in both the CNS and PNS (Pender and Sears, 1982, 1984; Pender, 1988*a*, *b;* Pender *et al.,* 1989, 1995; Chalk *et al., 1994a,b;* Abromson-Leeman *et al.,* 1995).

A number of studies have investigated whether multiple sclerosis patients have an increased frequency of cells reactive to myelin antigens, including PLP or MBP, in their blood or CSF. These studies have been performed using a variety of methodologies and antigen preparations, often on only small numbers of subjects, and the results have been inconsistent. Many of these studies have involved the production of long-term T-cell lines and clones. Only a minority of the studies have looked at the immediate proliferative response of peripheral blood mononuclear cells (PBMC); these studies have investigated responses to whole MBP (Lisak and Zweiman, 1977; Brinkman *et al.,* 1982; Johnson *et al.,* 1986; Vandenbark *et al.,* 1989; Trotter *et al.,* 1991; Kerlero de Rosbo *et al.,* 1993; Zhang *et al.,* 1993), MBP fragments (Baxevanis *et al.,* 1989), whole PLP and/or two synthetic PLP peptides (Johnson *et al.,* 1986; Trotter *et al.,* 1991; Kerlero de Rosbo *et al.,* 1993), and two quantitatively minor myelin components, MOG (myelin/oligodendrocyte glycoprotein) (Kerlero de Rosbo *et al.,* 1993) and MAG (myelin-associated glycoprotein) (Zhang *et al.,* 1993). These have produced diverse results with little concurrence.

Because there exists a correlation between the expression of the major histocompatibility complex (MHC) class II haplotypes, human leucocyte antigens (HLA) DR2, DW2 (HLA-DRB1*1501, –DQA1*0102 and –DQB1*0602), and susceptibility to multiple sclerosis (Stewart *et al.,* 1981; Hillert *et al.,* 1994), it is thought that the pathogenesis of multiple sclerosis may involve T cells that recognize the autoantigen(s) in the context of these MHC class II molecules. Antigens presented by MHC class II molecules are generally 12–25 amino acids in length (Rammensee *et al.,* 1995). Therefore, the aim of the current study was to assess the proliferative responses of freshly isolated PBMC from multiple sclerosis patients, healthy control subjects and patients with other neurological diseases to multiple short (12–20 amino acid residues) synthetic peptides of PLP and MBP, in order to determine if there is a correlation between multiple sclerosis and reactivity to these antigens.

Several PLP epitopes that are immunodominant in a variety of genetically diverse mouse strains (Greer *et al.,* 1996*a*) and in humans (Markovic-Plese *et al.,* 1995) have not previously been tested for their ability to stimulate freshly isolated human PBMC, and no previous studies have assessed reactivity to peptides within all four major isoforms of human MBP. The use of synthetic peptides is particularly warranted in the case of PLP which, because of its very hydrophobic nature, has a strong tendency to form high molecular weight aggregates that readily precipitate in the presence of salts. This makes the PLP protein a poor antigen in *in vitro* tissue culture studies, as the precipitated, flocculent PLP can inhibit cell–cell contact and subsequent cell activation. However, Markovic-Plese *et al.* (1995), using multiple *in vitro* stimulations with human PLP, have found that the protein can be processed by antigen-presenting cells into immunodominant epitopes within the 30–60 and 180–210 regions of the molecule. Peptides covering these regions are included in the current study.

## Methods

### *Patients and control subjects*

The subjects of this study consisted of 61 patients with clinically definite or laboratory-

supported definite multiple sclerosis (Poser *et al.*, 1983), 56 healthy control subjects and 52 patients with other neurological diseases. Of the multiple sclerosis patients, 28 had relapsing-remitting multiple sclerosis, 19 had secondary progressive multiple sclerosis and 14 had primary progressive multiple sclerosis. Multiple sclerosis patients had not received corticosteroids for at least 2 months prior to being studied. Patients with other neurological diseases had the following diagnoses: epilepsy (12), Parkinson's disease (seven), Wilson's disease (one), Huntington's chorea (one), brain tumour (three), cerebral aneurysm (two), intracranial arteriovenous malformation (one), cerebrovascular disease (five), hydrocephalus (one), neurofibromatosis type I (one), neurosarcoidosis (one), sleeping disorder (one), spinocerebellar degeneration (one), motor neuron disease (three), multifocal motor neuropathy (one), hereditary sensory neuropathy (one), Guillain-Barré syndrome (two), non-specified peripheral neuropathy (four), C8 nerve root lesion (one), intercostal nerve entrapment (one), myasthenia gravis (one) and proximal myopathy (one).

### *Tissue typing*

Genomic DNA was prepared from Epstein–Barr-virus-transformed lymphoblastoid cell lines from each individual by chloroform/phenol extraction. HLA-DR typing was carried out at the Queensland Institute of Medical Research, Brisbane, Australia, and HLA-DQ typing at the Department of Immunology, Westmead Hospital, Sydney, Australia. For both HLA-DR and -DQ typing, DNA was initially amplified by the polymerase chain reaction (PCR) using generic primer pairs. Specific HLA-DRB1 alleles were then determined by hybridization of the PCR products with a panel of sequence-specific oligonucleotides. HLA-DQA1 and - DQB1 alleles were determined by restriction fragment length polymorphism analysis of the PCR products.

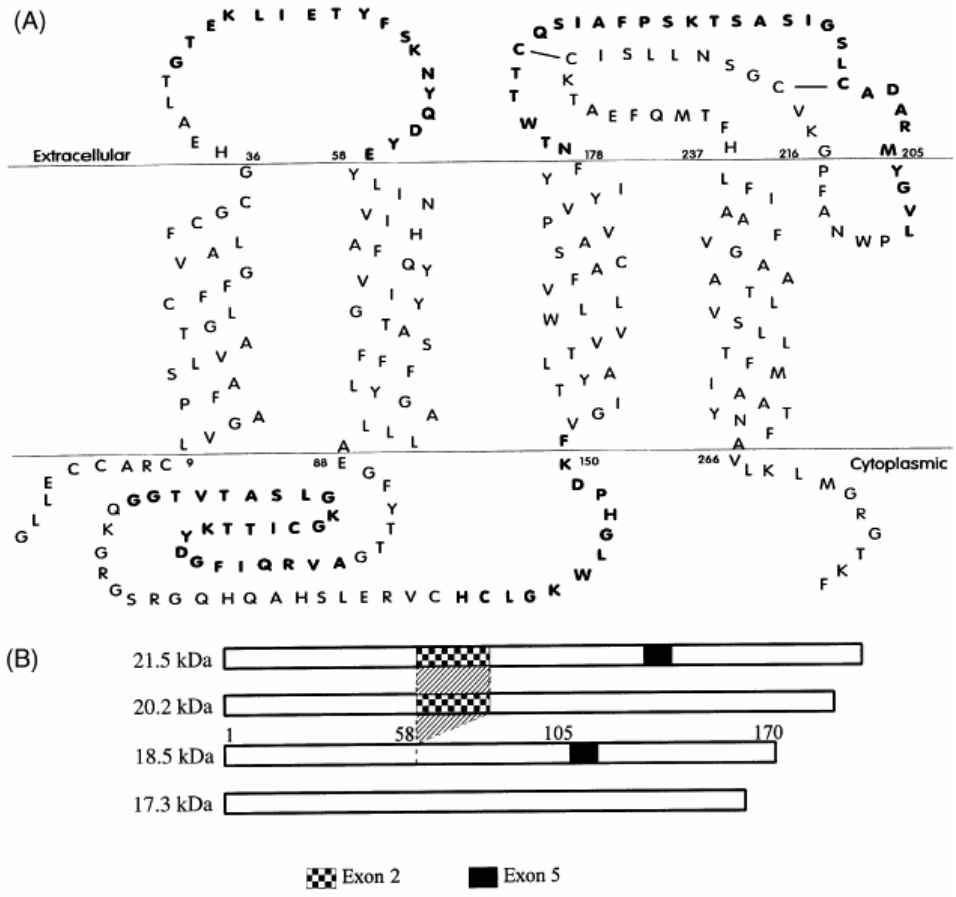



***Fig. 1.*** **(A)** The sequence of human PLP and its proposed orientation in the oligodendrocyte cell membrane (Greer *et al.*, 1996*b*) showing the peptides used in the current study in bold. **(B)** The four isoforms of human MBP, showing

the exon 2 insert (encoding 26 amino acids) in the 21.5 and 20.2 kDa isoforms, and exon 5 (encoding 11 amino acids) which is deleted in the 20.2 and 17.3 kDa isoforms.

### Antigens

Human PLP was prepared from human brain white matter as previously described (Greer *et al.*, 1996*a*). PLP peptides were prepared according to the human sequence (Hudson *et al.*, 1989) and were > 90% pure by HPLC analysis. The sequence of PLP showing the peptides used in the present study and the putative orientation of PLP in the myelin membrane is shown in Fig. 1A. $PLP_{41-58}$ (GTEKLIETYFSKNYQDYE) and $PLP_{95-114}$ (AVRQIFGDYKTTICGKGLSA) were synthesized at the Queensland Institute of Medical Research; $PLP_{178-191}$ (NTWTTCQSIAFPSK), $PLP_{184-199}$ (QSIAFPSKTSASIGSL) and $PLP_{190-209}$ (SKTSASIGSLCADARMYGVL) were synthesized by Auspep (Melbourne, Australia); and $PLP_{100-119}$ (FGDYKTTICGKGLSATVTGG) and $PLP_{139-151}$ (HCLGKWLGHPDKF) were the kind gift of Dr M. B. Lees, Shriver Center, Waltham, Mass., USA. $PLP_{100-119}$ and $PLP_{139-151}$ are water-soluble. The other PLP peptides are moderately hydrophobic, and were dissolved at a concentration of 5 mg/ml in 0.2 M acetic acid prior to dilution in tissue-culture medium for proliferation assays.

Human MBP was extracted from human brain according to the method of Deibler *et al.* (1972). Overlapping MBP peptides (numbered according to the human sequence and listed in Table 6) were synthesized (at the Queensland Institute of Medical Research) to represent the sequences of the 4 major isoforms (21.5 kDa, 20.2 kDa, 18.5 kDa and 17.3 kDa; shown in Fig. 1B) of human MBP (Roth *et al.*, 1987) and were > 80% pure. Peptides 5b and 5c represent the exon 2 insert (encoding 26 amino acids) in the 21.5 and 20.2 kDa isoforms. Peptide 10b covers the junctional zone formed by deletion of exon 5 (encoding 11 amino acids) in the 20.2 and 17.3 kDa isoforms. All MBP peptides are water-soluble.

### Proliferation assays

Heparinized peripheral blood (~60 ml) was collected by venepuncture from each subject after informed written consent had been obtained. PBMC were separated from blood by centrifugation through Histopaque (Sigma Chemical Co., St Louis, USA) and washed twice. The *in vitro* responses of PBMC were assayed in triplicate in 96-well round-bottomed microtitre plates. PBMC ($2\times10^5$) and medium (RPMI 1640 containing 10% heat-inactivated pooled human serum, 2 mM L-glutamine, 10 mM HEPES buffer and 50 μM 2-mercaptoethanol) with or without peptides were added to wells in a total volume of 200 μl. Initial studies were done using blood from five laboratory staff, all of whom had been exposed to both PLP and MBP for several years, and four multiple sclerosis patients to determine the optimal concentration of antigen to use in the main part of the study. Peptides were tested at concentrations ranging from 1 to 100 μg/ml. The concentrations used in the main part of the study (25 and 12.5 μg/ml final concentration for PLP peptides or 30 μg/ml final concentration for MBP peptides) were those at which the most responses occurred in the initial studies. Cultures were incubated for 6 days, with 0.5 μCi [$^3$H]thymidine being added during the last 18 h. Cultures were harvested and thymidine uptake was measured in c.p.m. (counts per minute) in a β-plate counter (LKB). The stimulation index (SI) was determined by the formula: SI = (mean c.p.m. of peptide-containing triplicate wells)/(mean c.p.m. of control triplicate wells, without peptide). The mean control ranged from 191 to 6071 c.p.m. A positive proliferative response for each sample was considered to be one in which the SI ≥ 3.0 and the mean from the control wells plus 3SD of the mean did not exceed the mean from the peptide-containing triplicate wells.

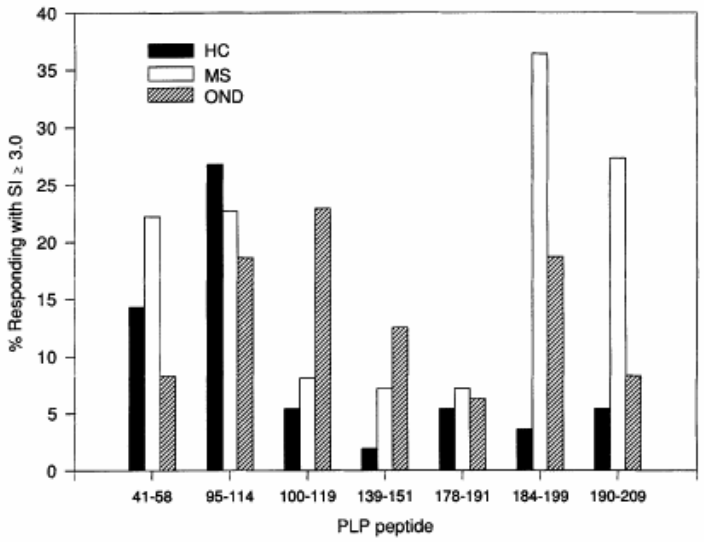
**TABLE 1**
Characteristics Of Multiple Sclerosis Patients and Control Subjects

| Parameter | PBMC tested against* | |
|---|---|---|
| Group | PLP peptides | MBP peptides |
| Number (female : male) | | |
| MS (whole group) | 55 (40 : 15) | 61 (45 : 16) |
| PP-MS | 11 (7 : 4) | 14 (9 : 5) |
| RR-MS | 26 (21 : 5) | 28 (22 : 6) |
| SP-MS | 18 (12 : 6) | 19 (14 : 5) |
| Healthy controls | 54 (32 : 22) | 56 (29 : 27) |
| OND | 48 (19 : 29) | 52 (23 : 29) |
| Mean age in years (range) | | |
| MS (whole group) | 44.3 (15–70) | 45.0 (15–70) |
| PP-MS | 49.5 (31–70) | 50.1 (31–70) |
| RR-MS | 37.9 (15–57) | 38.1 (15–57) |
| SP-MS | 50.4 (30–65) | 50.1 (30–65) |
| Healthy controls | 36.3 (15–61) | 35.7 (15–61) |
| OND | 49.5 (19–75) | 50.3 (16–75) |
| Mean years duration (range) | | |
| MS (whole group) | 11.0 (1–31) | 10.6 (1–28) |
| PP-MS | 5.6 (1–11) | 6.2 (1–14) |
| RR-MS | 8.8 (1–26) | 8.7 (1–26) |
| SP-MS | 17.6 (6–31) | 16.7 (6–28) |
| Healthy controls | Not applicable | Not applicable |
| OND | Not applicable | Not applicable |
| Mean EDSS score (range) | | |
| MS (whole group) | 5.4 (0–9.5) | 5.5 (0–9.5) |
| PP-MS | 5.9 (3.5–7.5) | 6.1 (3.5–7.5) |
| RR-MS | 3.6 (0–8.0) | 3.9 (0–8.0) |
| SP-MS | 7.3 (5.0–9.5) | 7.4 (5.0–9.5) |
| Healthy controls | Not applicable | Not applicable |
| OND | Not applicable | Not applicable |
| HLA-Dw2$^+$ | | |
| MS (whole group) | 80% | 80% |
| PP-MS | 90% | 87% |
| RR-MS | 69% | 68% |
| SP-MS | 89% | 94% |
| Healthy controls | 34% | 34% |
| OND | 36% | 37% |

*The peripheral blood mononuclear cells (PBMC) of some patients were only tested for reactivity to myelin proteolipid protein (PLP) or myelin basic protein (MBP) peptides as described in text. MS = multiple sclerosis; PP-MS = primary progressive multiple sclerosis; RR-MS = relapsing–remitting multiple sclerosis; SP-MS = secondary progressive multiple sclerosis; OND = other neurological diseases; EDSS = Kurtzke's Expanded Disability Status Scale; HLA-Dw2$^+$ = percentage of individuals expressing one or more of the alleles DRB1*1501, DQA1*0102 or DQB1*0602.

### *Generation and testing of short-term T-cell lines*

Short-term T-cell lines were prepared from three multiple sclerosis patients by incubation of PBMC ($4\times10^6$ cells per well) with 25 μg/ml of human PLP, $PLP_{184-199}$ or $PLP_{190-209}$ in 24-well plates. Lines were restimulated 10 days later with antigen plus irradiated (3000 rad) autologous PBMC and then tested 12–15 days later in proliferative assays. For these assays, $5\times10^4$ T cells were added to $10^5$ irradiated autologous PBMC and antigen in 96-well microtitre trays. Cultures were incubated for 4 days, with 0.5 μCi [$^3$H]thymidine being added during the last 18h. Cultures were harvested and thymidine uptake was measured as described above.




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 3 x 2 | $P = 0.144$ | $P > 0.5$ | $P = 0.014$ | $P = 0.108$ | $P > 0.5$ | $P < 0.001$ | $P = 0.002$ |
| MS vs HC | $P > 0.5$ | $P > 0.5$ | $P > 0.5$ | $P > 0.5$ | $P > 0.5$ | $P < 0.001$ | $P = 0.004$ |
| MS vs OND | $P = 0.095$ | $P > 0.5$ | $P = 0.081$ | $P > 0.5$ | $P > 0.5$ | $P = 0.075$ | $P = 0.025$ |
| OND vs HC | $P > 0.5$ | $P > 0.5$ | $P = 0.019$ | $P = 0.08$ | $P > 0.5$ | $P = 0.027$ | $P > 0.5$ |

***Fig. 2.*** Percentages of healthy control subjects (HC), and patients with multiple sclerosis (MS) and other neurological diseases (OND), responding with SI ≥ 3.0 to PLP peptides. The *P* values for comparison of the responses of the three groups together with those for the comparisons of the pairs of groups ($\chi 2$ analysis) are shown directly below the peptide to which they refer.

### *Statistical analysis*

Percentages of individuals making a positive proliferative response to peptides were compared using the $\chi^2$ test with Yates' correction applied as required. Mean SI values were compared using analysis of variance (ANOVA) to compare all the groups simultaneously, followed by Student's *t* test to compare the pairs of groups.

## Results

### *Characteristics of multiple sclerosis patients and control subjects*

The characteristics of multiple sclerosis patients and control subjects are detailed in Table 1. PBMC from all patients with other neurological diseases and all except one multiple sclerosis patient that were tested for reactivity to PLP peptides were concurrently tested for reactivity to the MBP peptides. Seven additional multiple sclerosis patients and four additional patients with other neurological diseases were tested for reactivity to MBP peptides, but not PLP peptides. In the healthy control group, three females were tested only for reactivity to PLP peptides, and five males were tested only for reactivity to MBP peptides. As has previously been found for other groups of multiple sclerosis patients, our multiple sclerosis patients were predominantly female, the mean age of onset of multiple sclerosis was higher for primary progressive multiple sclerosis than for relapsing-remitting multiple sclerosis and secondary progressive multiple sclerosis, and the percentage of patients expressing the HLA-Dw2 haplotype was increased in multiple sclerosis patients compared with control subjects.

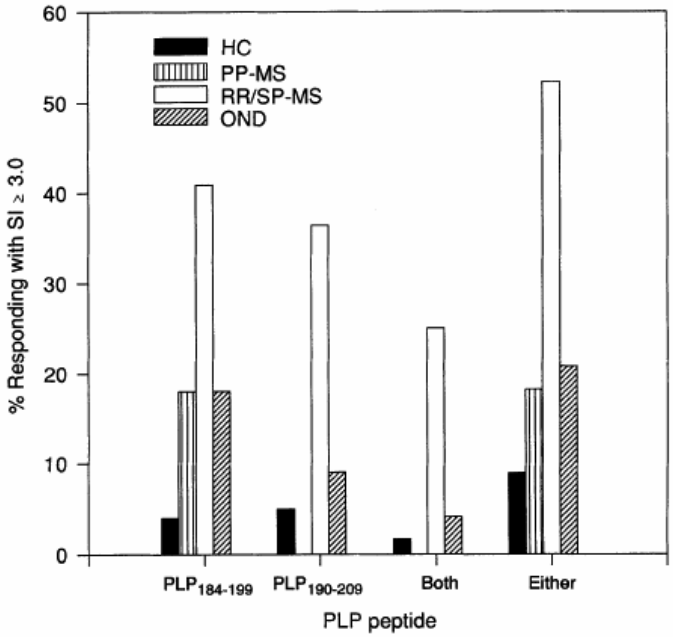
**TABLE 2**
Mean Stimulation Index (SI ± SE) of PBMC from Healthy Control Subjects, Multiple Sclerosis Patients and Patients with Other Neurological Diseases to PLP Peptides

| Peptide | Healthy controls | Multiple sclerosis | Other neurological diseases | *P*-value ANOVA* |
|---|---|---|---|---|
| $PLP_{41–58}$ | 1.8 ± 0.4 | 2.2 ± 0.3** | 1.6 ± 0.2 | 0.019 |
| $PLP_{95–114}$ | 2.9 ± 0.4 | 3.0 ± 0.9 | 2.5 ± 0.6 | 0.764 |
| $PLP_{100–119}$ | 1.6 ± 0.1 | 1.5 ± 0.1 | 2.5 ± 0.4 | 0.066 |
| $PLP_{139–151}$ | 1.2 ± 0.1 | 1.5 ± 0.1 | 2.0 ± 0.3 | 0.113 |
| $PLP_{178–191}$ | 1.5 ± 0.1 | 1.5 ± 0.2 | 1.9 ± 0.5 | 0.799 |
| $PLP_{184–199}$ | 1.7 ± 0.2 | 3.0 ± 0.6 | 2.2 ± 0.5 | 0.068 |
| $PLP_{190–209}$ | 1.4 ± 0.1 | 2.1 ± 0.2*** | 1.7 ± 0.2 | 0.012 |

*If $P < 0.05$ with ANOVA, then Student's *t* test was used to compare pairs of groups. **Mean SI of multiple sclerosis patients is significantly different from the mean SI of patients with other neurological diseases (Student's *t* test; $P = 0.037$), but not of healthy control subjects. ***Mean SI of multiple sclerosis patients is significantly different from the mean SI of healthy control subjects ($P = 0.003$), but not of patients with other neurological diseases.

### *Reactivity to PLP peptides*

Epitopes of PLP that are immunogenic in a variety of experimental animals cluster in three regions of the molecule (Greer *et al.,* 1996*a*). Peptides covering these regions (residues 40–60, 90–120 and 180–210), together with $PLP_{139-151,}$ which is an encephalitogenic epitope in SJL mice (Tuohy *et al.,* 1989), were tested for their ability to induce proliferative responses with SI ≥ 3.0 in freshly isolated PBMC collected from 55 multiple sclerosis patients, 54 healthy control subjects and 48 patients with other neurological diseases. Peptides from two of the PLP epitope clusters (residues 40–60 and 90–120) have previously been used to derive T-cell lines from multiple sclerosis patients' lymphocytes (Pelfrey *et al.,* 1993, 1994; Correale *et al.,* 1995; Markovic-Plese *et al.,* 1995; Ohashi *et al.,* 1995). In the present study, however, the percentages of multiple sclerosis patients with cells responding to peptides within these clusters were not significantly different from those of healthy control subjects or of patients with other neurological diseases (Fig. 2). However, there was a slight increase in the percentage of multiple sclerosis patients with cells responding to $PLP_{41-58,}$ and the mean SI of multiple sclerosis patients for $PLP_{41-58}$ was significantly higher than the mean SI of cells from patients with other neurological diseases, but not of those from healthy control subjects (Table 2). All three groups showed a relatively high percentage of subjects with cells responding to $PLP_{95-114}$ (Fig. 2) and the mean SI values for this peptide were high in all groups compared with values for other peptides (Table 2). This reactivity correlated strongly with the expression of the HLA-DRB1*1101 allele: 93% of all individuals expressing this allele had cells which responded to $PLP_{95-114,}$ and ~60% of 'responders' in each of the three groups expressed this allele. More patients with other neurological diseases responded to $PLP_{100-119}$ and $PLP_{139-151}$ than did healthy control subjects or multiple sclerosis patients; however, the reactivity in patients with other neurological diseases did not correlate strongly with any particular disease or with expression of a particular HLA-DR or –DQ allele. Few subjects from any group responded to $PLP_{178-191}$ (Fig. 2 and Table 2).




| 4 × 2 | *P* < 0.001 | *P* < 0.001 | *P* < 0.001 | *P* < 0.001 |
|---|---|---|---|---|
| RR/SP-MS vs HC | *P* < 0.001 | *P* < 0.001 | *P* = 0.002 | *P* < 0.001 |
| RR/SP-MS vs OND | *P* = 0.034 | *P* = 0.003 | *P* = 0.010 | *P* = 0.002 |
| RR/SP-MS vs PP-MS | *P* = 0.294 | *P* = 0.043 | *P* = 0.149 | *P* = 0.087 |
| PP-MS vs HC | *P* = 0.257 | *P* > 0.5 | *P* > 0.5 | *P* > 0.5 |
| PP-MS vs OND | *P* > 0.5 | *P* > 0.5 | *P* > 0.5 | *P* > 0.5 |
| OND vs HC | *P* = 0.032 | *P* > 0.5 | *P* > 0.5 | *P* = 0.169 |

***Fig. 3.*** Percentages of healthy control subjects (HC), and patients with primary progressive multiple sclerosis (PP-MS), relapsing–remitting or secondary progressive multiple sclerosis (RR/SP-MS), or other neurological diseases (OND), responding to $PLP_{184-199}$ and/or $PLP_{190-209}$. The *P* values are shown directly below the peptide(s) to which they refer ($\chi^2$ analysis).

In contrast, the percentage of multiple sclerosis patients with PBMC in their blood that responded to $PLP_{190-209}$ was significantly higher than the corresponding percentages of healthy control subjects and of patients with other neurological diseases (Fig. 2). The difference between multiple sclerosis patients and patients with other neurological diseases could not be explained by the predominance of males in the group of patients with other neurological diseases, as the percentage of male patients with other neurological diseases responding to this peptide was greater than the percentage of females. The percentage of multiple sclerosis patients responding to the overlapping peptide $PLP_{184-199}$ was also increased, but only the comparison with healthy control subjects reached statistical significance. Reactivity to these peptides was not restricted to individuals expressing any particular HLA-DR or –DQ allele. The majority of responders in the multiple sclerosis group (91%) expressed HLA-Dw2 alleles. However, only 53% of responders in the control groups expressed these alleles. The expression of DRB1*1501 in combination with DRB1*0401, DRB1*0404 or DRB1*0701 did, however, appear to be related to increased responses to $PLP_{184-199}$ and $PLP_{190-209}$. The mean SI value of multiple sclerosis patients for $PLP_{190-209}$ was significantly higher than that of healthy control subjects, but not of patients with other neurological diseases (Table 2). The mean SI of multiple sclerosis patients for $PLP_{184-199}$ was increased compared with that of healthy control subjects and that of patients with other neurological diseases, but comparison of the three groups by ANOVA did not reach statistical significance.

Within the multiple sclerosis group, reactivity to $PLP_{184-199}$ and $PLP_{190-209}$ occurred most frequently in patients with relapsing–remitting multiple sclerosis or secondary progressive multiple sclerosis, who were grouped together (relapsing-remitting/secondary-progressive multiple sclerosis) (Fig. 3) because the results were similar in the two groups. Reactivity to

these peptides was found significantly more frequently in patients with relapsing-remitting/secondary progressive multiple sclerosis than in healthy control subjects or patients with other neurological diseases (Fig. 3). Few patients with primary progressive multiple sclerosis reacted to these peptides (Fig. 3), and the mean SI values of primary progressive multiple sclerosis patients for these peptides were significantly lower than those of patients with relapsing–remitting/secondary progressive multiple sclerosis (Table 3). The highest frequency of reactivity to $PLP_{184-199}$ and/or $PLP_{190-209}$ occurred in those relapsing–remitting/secondary progressive multiple sclerosis patients who had a disease duration of 6–15 years (Fig. 4). Fifteen of 19 patients with this duration of disease responded to one or both peptides. The mean SI values of patients with relapsing–remitting/secondary progressive multiple sclerosis peaked in the 11–15 years duration group, at which time the mean SI values were significantly different from those of both healthy control subjects and patients with other neurological diseases (Table 4). The differences between primary progressive multiple sclerosis patients and relapsing–remitting/secondary progressive multiple sclerosis patients are unlikely to be solely due to the shorter disease duration in primary progressive multiple sclerosis patients, as the mean SI values for primary progressive multiple sclerosis patients with a disease duration of 6–11 years were substantially lower than those for patients with relapsing–remitting/secondary progressive multiple sclerosis with a duration of 6–10 years (Table 4).

To determine whether the $PLP_{184-199}$ and $PLP_{190-209}$ peptides can be naturally processed from the whole PLP molecule, short-term T-cell lines specific for whole human PLP, $PLP_{184-199}$ or $PLP_{190-209}$ were prepared from three multiple sclerosis patients who responded to these peptides in the initial assays. The T-cell lines were then tested for their ability to proliferate in response to the three antigens. Short-term PLP-specific lines from multiple sclerosis patient 22 and multiple sclerosis patient 45 showed increased responses to $PLP_{184-199}$ and $PLP_{190-209;}$ however, the PLP-specific line from multiple sclerosis patient 35 did not respond significantly to either peptide (Table 5). All of the lines specific for $PLP_{184-199}$ responded significantly to all three antigens. In contrast, the response of $PLP_{190-209}$-specific lines was variable, with lines from multiple sclerosis patient 22 and multiple sclerosis patient 45 responding significantly only to $PLP_{190-209,}$ whereas the line from multiple sclerosis patient 35 responded to whole PLP and $PLP_{184-199}$, in addition to $PLP_{190-209}$. As predicted from previous experience with PLP (as described in the Introduction), whole PLP was unable to induce responses of the same magnitude as the peptides, even when the T-cell line was generated against the whole molecule. Nevertheless, there were significant responses to whole PLP by at least one peptide-specific line from each of the three multiple sclerosis patients, indicating that whole PLP can be naturally processed into epitopes within the 184-209 region of the molecule. The results suggest that there are at least two dominant naturally processed epitopes of PLP, one in the overlapping 190–199 region and one present only in $PLP_{184-199}$.

**TABLE 3**

Mean Stimulation Index (SI ± SE) of PBMC From Primary Progressive Multiple Sclerosis (PP-MS) Patients, Relapsing-Remitting or Secondary Progressive Multiple Sclerosis (RR/SP-MS) Patients, Healthy Controls And Patients With Other Neurological Diseases

| Peptide | Healthy controls ($n$ = 54) | Other neurological diseases ($n$ = 48) | PP-MS ($n$ = 11) | RR/SP-MS ($n$ = 44) | $P$-value ANOVA* |
|---|---|---|---|---|---|
| $PLP_{184–199}$ | 1.7 ± 0.2 | 2.2 ± 0.5 | 1.7 ± 0.4 | 3.3 ± 0.6** | <0.001 |
| $PLP_{190–209}$ | 1.4 ± 0.1 | 1.7 ± 0.2 | 1.3 ± 0.1 | 2.3 ± 0.3*** | <0.001 |

*ANOVA was used to compare PP-MS and RR/SP-MS patients, patients with other neurological diseases and healthy controls. **Mean SI of RR/SP-MS patients is significantly different from the mean SI of healthy control subjects (Student's $t$ test; $P$ = 0.018), and of PP-MS patients ($P$ = 0.033), but not of patients with other neurological diseases. ***Mean SI of RR/SP-MS patients is significantly different from the mean SI of healthy control subjects ($P$ = 0.002) and of patients with other neurological diseases ($P$ = 0.05), and from that of PP-MS patients ($P$ < 0.001).

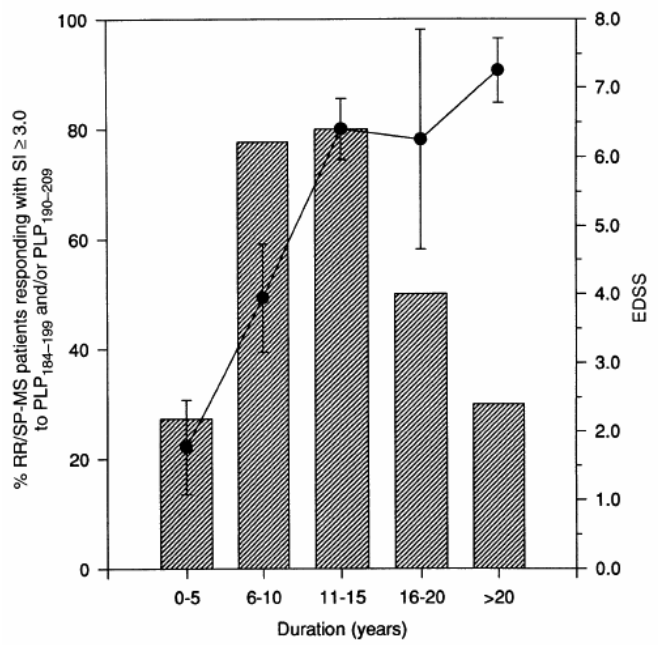



***Fig. 4.*** Relationship between responsiveness of PBMC to $PLP_{184-199}$ and/or $PLP_{190-209}$ and duration of relapsing-remitting or secondary progressive multiple sclerosis (RR/SP-MS) and EDSS score (filled circles). The number of individuals for each duration was: 0–5 years, $n = 11$; 6–10 years, $n = 9$; 11–15 years, $n = 10$; 16–20 years, $n = 4$; >20 years, $n = 10$.

**TABLE 4**

Mean Stimulation Index (SI ± SE) Of PBMC from Patients With Relapsing-Remitting Or Secondary Progressive Multiple Sclerosis (RR/SP-MS) and Primary Progressive Multiple Sclerosis (PP-MS) of Different Disease Durations

| Peptide | HC ($n$ =54) | OND ($n$ =48) | RR/SP-MS duration (years) | | | | | PP-MS duration (years) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 0–5 ($n$ =11) | 6–10 ($n$ = 9) | 11–15 ($n$ = 10) | 16–20 ($n$ = 4) | >20 ($n$ = 10) | 0–5 ($n$ = 5) | 6–11 ($n$ = 6) |
| $PLP_{184–199}$ | 1.7 ± 0.2 | 2.2 ± 0.5 | 1.7 ± 0.3 | 2.8 ± 0.6* | 7.5 ± 2.7*** | 2.1 ± 0.7 | 1.8 ± 0.4 | 1.9 ± 0.8 | 1.6 ± 0.5 |
| $PLP_{190–209}$ | 1.4 ± 0.1 | 1.7 ± 0.2 | 1.5 ± 0.3 | 2.2 ± 0.5** | 3.9 ± 0.7*** | 2.2 ± 0.7* | 1.5 ± 0.5 | 1.1 ± 0.2 | 1.4 ± 0.1 |

When ANOVA was used to compare healthy control (HC) subjects, patients with other neurological diseases (OND), the five groups of RR/SP-MS patients and the two groups of PP-MS patients, then $P < 0.000035$ for each peptide. * $P < 0.048$ when compared with HC subjects (in Student's $t$ test), but not significant compared with OND patients. ** $P = 0.003$ when compared with HC subjects, but not significant compared with OND patients. *** $P < 0.002$ compared with both HC subjects and OND patients.

### *Reactivity to MBP and MBP peptides*

The responses of PBMC from 61 multiple sclerosis patients, 56 healthy control subjects and 52 patients with other neurological diseases to 19 overlapping 20-amino acid-residue peptides of MBP were also tested in proliferation assays. There were no significant differences between the percentages of multiple sclerosis patients and healthy control subjects responding with SI ≥ 3.0 to the MBP peptides, except for $MBP_{11–30,}$ which elicited a response in a significantly higher percentage of healthy control subjects than of multiple sclerosis patients (Table 6). PBMC from multiple sclerosis patients responded most frequently to peptides 5b, $MBP_{82-100}$, $MBP_{91-110}$ and $MBP_{101-120;}$ however, similar percentages of PBMC from healthy control subjects responded to these peptides. In the multiple sclerosis group, 83%, 85%, 85% and 100%, respectively of patients responding to peptides 5b, $MBP_{82-100}$, $MBP_{91-110}$ and $MBP_{101-120}$ expressed

at least one HLA-Dw2 allele. However, in the control group, only 36%, 33%, 47% and 42%, respectively of responders to peptides 5b, $MBP_{82-100}$, $MBP_{91-110}$ and $MBP_{101-120}$ expressed HLA-Dw2 alleles. Interestingly, the percentages of PBMC from patients with other neurological diseases responding to peptides 5b and $MBP_{82-100}$ were significantly reduced compared with those of healthy control subjects and multiple sclerosis patients (Table 6). These differences could not be explained by the predominance of males in the group of patients with other neurological diseases. There was no obvious relationship between responses to peptide 5b and the duration of multiple sclerosis; however, the highest percentage of responders to $MBP_{82-100}$ was in the group with disease duration of ≤ 5 years (Fig. 5). Analysis of the responses of the primary progressive multiple sclerosis, relapsing–remitting multiple sclerosis and secondary progressive multiple sclerosis subgroups did not reveal any differences in reactivity to the MBP peptides among the groups. The percentage of multiple sclerosis patients reacting to whole MBP was intermediate between the percentages of healthy control subjects and patients with other neurological diseases, but the differences were not significant (Table 6). The mean SI values for the MBP peptides and MBP (Table 7) showed the same pattern of reactivity as that indicated by the $\chi^2$ analysis.

## Discussion

This study indicates that there is increased reactivity against $PLP_{184-199}$ and $PLP_{190-209}$ in relapsing-remitting multiple sclerosis and secondary-progressive multiple sclerosis patients, but not primary-progressive multiple sclerosis patients, compared with healthy control subjects and patients with other neurological diseases (Fig. 3). Although stimulation assays with synthetic peptides can potentially introduce a bias for cryptic epitopes, we have shown that $PLP_{184-199}$-specific T cells and $PLP_{190-209}$-specific T cells can respond to whole PLP and vice versa, indicating that these epitopes of PLP can be processed naturally from whole PLP by antigen-presenting cells of multiple sclerosis patients. Autoimmune responses to epitopes within the same region of PLP (residues 178–209) mediate experimental autoimmune encephalomyelitis (EAE) in a variety of mice of different genetic backgrounds (Greer *et al.*, 1992, 1996*a*). We did not find a correlation between PBMC proliferative responses to MBP peptides and multiple sclerosis. However, the differences in responses of multiple sclerosis patients and patients with other neurological diseases to MBP peptides do raise some questions regarding regulation of responses to MBP, which will be discussed below.

**TABLE 5**

Reactivity Of Short-Term T-Cell Lines To Whole Myelin Proteolipid Protein (PLP), $PLP_{184–199}$ or $PLP_{190–209}$

| Patient | HLA haplotype | | | SI in initial proliferation assay on PBMC | | Antigen | Reactivity of the line (c.p.m.) according to antigen used to generate line | | |
|---|---|---|---|---|---|---|---|---|---|
| | DRB1 | DQA1 | DQB1 | $PLP_{184–199}$ | $PLP_{190–209}$ | | PLP | $PLP_{184–199}$ | $PLP_{190–209}$ |
| MS22 | 0401, 1501 | 0102, 0301 | 0301, 0602 | 4.9 | 1.7 | – | 1529 ± 1121 | 1492 ± 435 | 1761 ± 630 |
| | | | | | | PLP | 4493 ± 1343 | 6626 ± 2989* | 2187 ± 596 |
| | | | | | | $PLP_{184–199}$ | 5071 ± 426* | 46,742 ± 4994* | 3143 ± 1054 |
| | | | | | | $PLP_{190–209}$ | 5057 ± 621* | 18,940 ± 2578* | 6940 ± 540* |
| MS35 | 0701, 1501 | 0102, 0201 | 0201, 0602 | 3.1 | 8.2 | – | 335 ± 40 | 555 ± 487 | 382 ± 34 |
| | | | | | | PLP | 553 ± 35 | 3313 ± 774* | 1884 ± 390* |
| | | | | | | $PLP_{184–199}$ | 460 ± 36 | 2498 ± 461* | 2690 ± 786* |
| | | | | | | $PLP_{190–209}$ | 617 ± 58 | 4430 ± 612* | 35,831 ± 958* |
| MS45 | 0401, 1101 | 0301, 0501 | 0301, 0301 | 3.5 | 5.8 | – | 1884 ± 953 | 521 ± 134 | 603 ± 100 |
| | | | | | | PLP | 3840 ± 1121 | 1628 ± 329* | 830 ± 67 |
| | | | | | | $PLP_{184–199}$ | 10,911 ± 2402* | 11,362 ± 1568* | 1285 ± 143 |
| | | | | | | $PLP_{190–209}$ | 6346 ± 1453* | 2437 ± 170* | 7014 ± 396* |

*Positive responses.

**TABLE 6**
Percentage Of Subjects Responding To Myelin Basic Protein (MBP) and MBP Peptides

| MBP peptide | | Sequence | Percentage with SI ≥ 3.0 | | |
|---|---|---|---|---|---|
| No. | According to human 18.5-kDa isoform | | HC | MS | OND |
| 1 | 1–20 | ASQKRPSQRHGSKYLATAST | 7.0 | 3.3 | 0 |
| 2 | 11–30 | GSKYLATASTMDHARHGFLP | 26.3* | 3.3 | 3.9 |
| 3 | 21–40 | MDHARHGFLPRHRDTGILDS | 12.3 | 4.9 | 7.7 |
| 4 | 31–50 | RHRDTGILDSIGRFFGGDRG | 14.0 | 3.3 | 3.9 |
| 5 | 41–60 | IGRFFGGDRGAPKRGSGKDS | 19.3 | 13.1 | 9.6 |
| 5b | (49–58) + (exon 2, 1–10) | RGAPKRGSGKVPWLKPGRSP | 17.5 | 19.7 | 1.9** |
| 5c | (exon 2, 1–20) | VPWLKPGRSPLPSHARSQPG | 12.3 | 8.2 | 7.7 |
| 6 | 51–70 | APKRGSGKDSHHPARTAHYG | 12.3 | 9.8 | 7.7 |
| 7 | 61–80 | HHPARTAHYGSLPQKSHGRT | 21.1 | 11.5 | 11.5 |
| 8 | 71–90 | SLPQKSHGRTQDENPVVHFF | 8.8 | 3.3 | 5.8 |
| 9 | 82–100 | DENPVVHFFKNIVTPRTPP | 24.6 | 23.0 | 5.8** |
| 10 | 91–110 | KNIVTPRTPPPSQGKGRGLS | 24.6 | 18.0 | 9.6 |
| 10b | (96–105) + (117–126) | PRTPPPSQGKGAEGQRPGFG | 5.3 | 8.2 | 1.9 |
| 11 | 101–120 | PSQGKGRGLSLSRFSWGAEG | 14.0 | 19.7 | 9.6 |
| 12 | 111–130 | LSRFSWGAEGQRPGFGYGGR | 15.8 | 16.4 | 13.5 |
| 13 | 122–140 | RPGFGYGGRASDYKSAHKG | 8.8 | 3.3 | 7.7 |
| 14 | 131–150 | ASDYKSAHKGFKGVDAQGTL | 15.8 | 6.6 | 9.6 |
| 15 | 141–160 | FKGVDAQGTLSKIFKLGGRD | 21.1 | 13.1 | 7.7 |
| 16 | 151–170 | SKIFKLGGRDSRSGSPMARR | 8.8 | 11.5 | 7.7 |
| MBP | – | | 35.1 | 24.6 | 17.3 |

The 3×2 $\chi 2$ analysis was significant ($P < 0.05$) for peptides 2, 4, 5b, and 9, but not for the other peptides. *Percentage of healthy control subjects (HC) responding to peptide is significantly different from the percentages of both multiple sclerosis patients (MS) ($P < 0.001$, $\chi 2$) and patients with other neurological diseases (OND) ($P = 0.002$) responding to the same peptide. **Percentage of OND patients responding to peptide is significantly different from the percentages of both MS patients and healthy control subjects responding to the same peptide ($P < 0.02$).

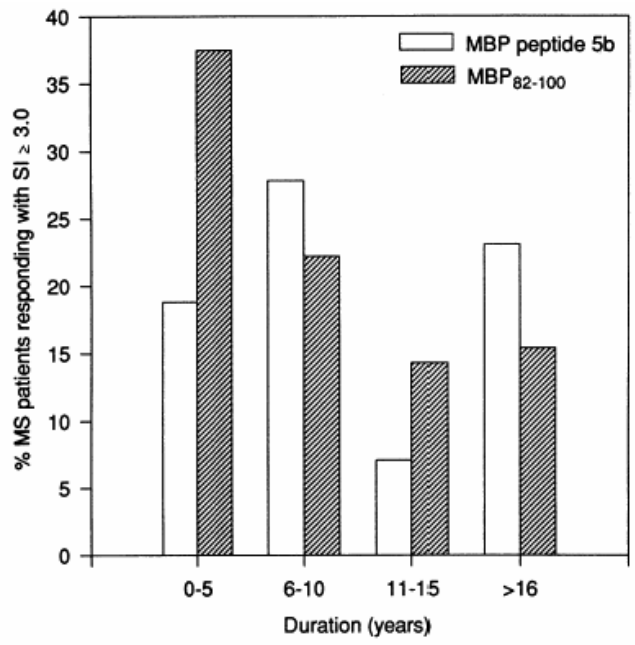



***Fig. 5.*** Relationship between responsiveness to MBP peptide 5b and $MBP_{82–100}$ and duration of multiple sclerosis. The number of individuals for each duration was: 0–5 years, $n = 16$; 6–10 years, $n = 18$; 11–15 years, $n = 14$; >16 years, $n = 13$.

### *Autoreactivity to PLP*

Three previous studies have investigated proliferative responses of PLP-specific lymphocytes in the blood of multiple sclerosis patients. Two of these studies reported no differences between multiple sclerosis patients and control subjects (Johnson *et al.*, 1986; Kerlero de Rosbo *et al.*, 1993), whereas Trotter *et al.* (1991) showed a difference between the responses of chronic

progressive (primary progressive + secondary progressive) multiple sclerosis patients and healthy control subjects to whole PLP. None of these studies investigated the response to peptides within the 180–210 region of the molecule. In the two studies (Johnson *et al.,* 1986; Kerlero de Rosbo *et al.,* 1993) that found no differences between multiple sclerosis patients and control subjects, bovine PLP was used, whereas in the study of Trotter *et al.* (1991) human PLP was used. PLP is a highly conserved molecule; there is < 1% difference in PLP among mammalian species, and murine and human PLP are completely identical (Macklin *et al.,* 1987; Hudson *et al.,* 1989). Bovine PLP, however, differs from human PLP at residues 88, 188 and 198 (Laursen *et al.,* 1984). These differences could affect the ability of human T cells to respond to bovine PLP, particularly since two of these differences occur in the region of the molecule that we have identified in the present study as being a target of autoreactive cells in multiple sclerosis. It is known that one of the murine encephalitogenic epitopes within the 180–209 region, $PLP_{178–191,}$ is encephalitogenic in SJL mice only when synthesized according to the murine sequence; it is not encephalitogenic when synthesized according to the bovine sequence (Greer *et al.,* 1997). In addition, in this murine model, mice immunized with murine PLP or the murine peptide respond only to murine PLP/peptide in proliferation assays; there is no cross-reactivity with the bovine peptide. Thus, if a similar situation occurs in humans, responses to epitopes in this region could be missed if bovine PLP is used as the stimulating antigen.

Interestingly, Trotter *et al.* (1991) found that the percentage of patients with early (disease duration of ≤ 2 years) and low-disability (EDSS < 3.0) relapsing–remitting multiple sclerosis reacting to PLP was lower than that of patients with more severe progressive disease. This is in agreement with the present study, in which we found that the greatest reactivity to $PLP_{184–199}$ and/or $PLP_{190–209}$ occurred in relapsing–remitting multiple sclerosis and secondary progressive multiple sclerosis patients with a disease duration of 6–15 years. The low frequency of reactivity to PLP peptides in patients with a short disease duration may indicate that the 184–209 region of PLP is not the target of the initial autoimmune attack in most cases of multiple sclerosis. Reactivity to this region of PLP might develop only after substantial myelin damage has occurred. Alternatively, the frequency of specific lymphocytes in the periphery may have been low if the disease was quiescent when PBMC were collected.

**TABLE 7**

Mean SI ± SE Of PBMC of Healthy Controls, Multiple Sclerosis Patients and Patients With Other Neurological Diseases For MBP and MBP Peptides

| MBP peptide | | Mean SI ± SE | | | *P*-value (ANOVA) |
|---|---|---|---|---|---|
| No. | According to human 18.5 kDa isoform | HC | MS | OND | |
| 1 | 1–20 | 1.5 ± 0.2* | 1.2 ± 0.1 | 1.1 ± 0.1 | 0.041 |
| 2 | 11–30 | 2.1 ± 0.3* | 1.3 ± 0.2 | 1.3 ± 0.2 | 0.008 |
| 3 | 21–40 | 1.6 ± 0.2 | 1.9 ± 0.7 | 1.3 ± 0.1 | 0.563 |
| 4 | 31–50 | 1.6 ± 0.2 | 1.2 ± 0.1 | 1.3 ± 0.1 | 0.098 |
| 5 | 41–60 | 2.2 ± 0.3* | 1.5 ± 0.2 | 1.4 ± 0.2 | 0.019 |
| 5b | (49–58) + (exon 2, 1–10) | 1.8 ± 0.2 | 1.8 ± 0.2 | 1.1 ± 0.1** | 0.005 |
| 5c | (exon 2, 1–20) | 1.6 ± 0.2 | 1.9 ± 0.4 | 1.8 ± 0.6 | 0.926 |
| 6 | 51–70 | 1.7 ± 0.3 | 1.3 ± 0.2 | 1.3 ± 0.1 | 0.150 |
| 7 | 61–80 | 2.1 ± 0.3 | 1.6 ± 0.3 | 1.6 ± 0.2 | 0.257 |
| 8 | 71–90 | 1.3 ± 0.2 | 1.3 ± 0.1 | 1.3 ± 0.1 | 0.908 |
| 9 | 82–100 | 2.7 ± 0.4 | 2.1 ± 0.2 | 1.5 ± 0.2** | 0.029 |
| 10 | 91–110 | 2.6 ± 0.4 | 2.5 ± 0.7 | 2.1 ± 0.7 | 0.831 |
| 10b | (96–105) + (117–126) | 1.2 ± 0.2 | 1.4 ± 0.3 | 0.9 ± 0.1 | 0.236 |
| 11 | 101–120 | 2.3 ± 0.7 | 2.2 ± 0.4 | 1.6 ± 0.3 | 0.575 |
| 12 | 111–130 | 2.0 ± 0.4 | 1.8 ± 0.2 | 1.6 ± 0.2 | 0.544 |
| 13 | 122–140 | 1.2 ± 0.1 | 1.4 ± 0.5 | 1.3 ± 0.3 | 0.826 |
| 14 | 131–150 | 1.6 ± 0.2 | 1.4 ± 0.3 | 1.3 ± 0.1 | 0.444 |
| 15 | 141–160 | 2.7 ± 0.5* | 1.8 ± 0.2 | 1.6 ± 0.3 | 0.048 |
| 16 | 151–170 | 1.6 ± 0.2 | 1.5 ± 0.2 | 1.3 ± 0.1 | 0.368 |
| | MBP | 3.0 ± 0.4 | 2.2 ± 0.2 | 2.3 ± 0.3 | 0.118 |

*Mean SI of healthy control (HC) subjects is significantly different from mean SI of multiple sclerosis (MS) patients (Student's *t* test; $P < 0.049$) and patients with other neurological diseases (OND) ($P < 0.021$). **Mean SI of OND patients is significantly different from mean SI of MS patients ($P < 0.04$) and HC subjects ($P < 0.002$).

The association between multiple sclerosis and the expression of HLA-DRB1*1501, –DQA1*0102 and –DQB1*0602 (Dw2) alleles (Hillert *et al.,* 1994) suggests that the target antigen(s) in multiple sclerosis may have a high affinity for these molecules, in the same way that certain self peptides preferentially bind to HLA molecules associated with the autoimmune disease pemphigus vulgaris (Wucherpfennig *et al.,* 1995). The majority of relapsing-remitting multiple sclerosis and secondary progressive multiple sclerosis patients reacting to $PLP_{184–199}$ or $PLP_{190–209}$ expressed these multiple sclerosis-associated alleles. However, our results suggest that the development of reactivity to $PLP_{184–199}$ and/or $PLP_{190–209}$ may be related to the presence of other alleles in the genotype, in particular DRB1*0401, DRB1*0404, or DRB1*0701 (or other alleles in linkage disequilibrium with them). The most common genotype encountered in the group of multiple sclerosis patients tested in this study, HLA-DRB1*0301,1501, did not correlate with reactivity to the two peptides. Previous binding assays have shown that $PLP_{180–199}$ and $PLP_{190–209}$ bind with relatively high affinity to a variety of murine (Greer *et al.,* 1996*a*) and human class II MHC molecules, including HLA-DRB1*1501, DR4 and DRB4 (DR53), but not DR3 (Markovic-Plese *et al.,* 1995). Interestingly, Ito *et al.* (1996) have recently shown that transgenic mice expressing the human HLA-DR4 molecule are susceptible to induction of EAE with $PLP_{175–192,}$ an epitope overlapping $PLP_{184–199.}$ Markovic-Plese *et al.* (1995) also showed that $PLP_{184–199}$ is a dominant region recognized by human T cells (control and multiple sclerosis) that have been stimulated several times *in vitro* with PLP (Markovic-Plese *et al.,* 1995). We have also found that whole-PLP-stimulated T-cell lines can recognize both $PLP_{184–199}$ and $PLP_{190–209}$ and, in addition, that T-cell lines specific for these peptides can recognize the whole PLP molecule (Table 5). The specific epitopes recognized will depend largely on the particular HLA genotype of the individual. These results clearly demonstrate that the intact PLP molecule can be naturally processed into epitopes contained within the $PLP_{184–199}$ or $PLP_{190–209}$ peptides. These features of the immune response to PLP may allow susceptible individuals to develop increased reactivity to PLP, once myelin breakdown has occurred.

As already mentioned, PBMC from most of the primary progressive multiple sclerosis patients did not respond to $PLP_{184–199}$ or $PLP_{190–209.}$ Several studies have suggested that primary progressive multiple sclerosis differs from relapsing–remitting and secondary progressive multiple sclerosis (Confavreux *et al.,* 1980; Verjans *et al.,* 1983; Larsen *et al.,* 1985; Poser *et al.,* 1986; Olerup *et al.,* 1989; Thompson *et al.,* 1991; Revesz *et al.,* 1994). Both histological (Revesz *et al.,* 1994) and MRI (Thompson *et al.,* 1991) findings indicate that there is less inflammation in primary progressive multiple sclerosis than in the other forms of multiple sclerosis. It has also been suggested that expression of certain HLA genes, in addition to the Dw2 susceptibility alleles, may predispose towards primary progressive multiple sclerosis (Olerup *et al.,* 1989), although this evidence is inconclusive. Furthermore, primary-progressive multiple sclerosis and relapsing–remitting/secondary progressive multiple sclerosis also differ in the age of onset (Confavreux *et al.,* 1980; Verjans *et al.,* 1983), initial symptoms (Confavreux *et al.,* 1980; Larsen *et al.,* 1985) and prognosis with regard to disability (Confavreux *et al.,* 1980; Verjans *et al.,* 1983) and mortality (Poser *et al.,* 1986). Our present results support the notion that primary progressive multiple sclerosis differs from relapsing–remitting multiple sclerosis and secondary progressive multiple sclerosis.

### *Autoreactivity to MBP*

Some previous studies of the PBMC proliferative responses to MBP or MBP fragments have shown significantly higher reactivity to MBP in multiple sclerosis patients than in normal control subjects or patients with other neurological diseases (Lisak and Zweiman, 1977; Brinkman *et al.,* 1982; Baxevanis *et al.,* 1989; Vandenbark *et al.,* 1989), but other studies have not (Johnson *et al.,* 1986; Kerlero de Rosbo *et al.,* 1993). Zhang *et al.* (1994) have reported that there is an increased frequency of IL-2-responsive T cells specific for MBP in peripheral blood and CSF of multiple sclerosis patients. In the present study, the proportion of multiple

sclerosis patients reacting to whole MBP was intermediate between the proportions of healthy control subjects and patients with other neurological diseases, but the differences were not significant.

In the present study, the peptides that were recognized most often by PBMC from multiple sclerosis patients were $MBP_{82–100}$, $MBP_{91–110}$, $MBP_{101–120}$ and peptide 5b which covers a region expressed in the 21.5 and 20.2 kDa isoforms of MBP. Previously, we (Pender *et al.,* 1996) and others (Martin *et al.,* 1990; Ota *et al.,* 1990; Zhang *et al.,* 1992) have found that $MBP_{82–100}$ (which is the same sequence as the peptide numbered $MBP_{84–102}$ by Ota *et al.,* 1990) is an immunodominant region, as T cells stimulated with whole MBP frequently react with peptides in this region. Valli *et al.* (1993) showed that $MBP_{82–100}$ is promiscuous in its ability to bind with high affinity to many different HLA-DR molecules. In contrast, they found that $MBP_{101–120}$ binds with only low affinity to several HLA-DR molecules, including HLA-DRB1*1501. Not all individuals expressing HLA-Dw2 alleles reacted to $MBP_{82–100}$, $MBP_{91–110}$, $MBP_{101–120}$, and peptide 5b but, of those multiple sclerosis patients who did react, nearly all expressed Dw2.

Similar proportions of healthy control subjects and multiple sclerosis patients had PBMC that recognized $MBP_{82–100}$, $MBP_{91–110}$, $MBP_{101–120}$ and peptide 5b, but the proportions of patients with other neurological diseases responding to these peptides were considerably lower. We have suggested that multiple sclerosis might result from an inability to delete autoreactive T cells in the CNS (Tabi *et al.,* 1995). One possible explanation for our findings is that, in healthy control subjects, these MBP-reactive cells make a primary response to the peptides *in vitro;* in patients with other neurological diseases, in whom some damage to the CNS or PNS has occurred, the T cells have already encountered the MBP peptides *in vivo* and have been deleted in the nervous system, as occurs during spontaneous recovery from EAE (Tabi *et al.,* 1994, 1995); whereas, in multiple sclerosis patients, these T cells have not been deleted, even after repeated exposure to antigen *in vivo.* The same pattern of responses was not observed with all PLP peptides. However, this may be due to the absence of PLP in PNS myelin, and a consequent lack of deletion of PLP-reactive cells in patients with other neurological diseases of the PNS. Further study of the activation status of the responding cells from healthy control subjects, multiple sclerosis patients, and patients with other neurological diseases to certain MBP and PLP peptides will be required to resolve this issue. The lack of increased reactivity to MBP peptides in patients with multiple sclerosis might have been anticipated from the fact that EAE induced by immunization with MBP (or whole CNS tissue), or by the passive transfer of MBP-sensitized lymphocytes, is characterized by major involvement of the proximal PNS (Pender and Sears, 1982, 1984; Pender, 1988*a*, *b;* Pender *et al.,* 1989, 1995; Chalk *et al.,* 1994*a*, *b;* Abromson-Leeman *et al.,* 1995), which does not occur in typical multiple sclerosis.

## Conclusions

In the present study we have demonstrated increased T-cell reactivity to two overlapping PLP peptides in patients with relapsing–remitting or secondary progressive multiple sclerosis, but not primary progressive multiple sclerosis. Both of the peptides can be processed naturally from the intact PLP molecule. The region of PLP that is the target of this autoreactivity is encephalitogenic in a wide variety of mice of different genetic backgrounds. It is also a region in which species differences in the PLP sequence occur, and this may account for the failure of two previous studies, which used bovine rather than human PLP, to identify PLP as a target of autoreactive cells in the blood of multiple sclerosis patients. The strongest and most frequent reactivity to these PLP peptides occurred in those relapsing– remitting multiple sclerosis and secondary progressive multiple sclerosis patients with multiple sclerosis of 6–15 years duration and with moderate to severe disability. In contrast, multiple sclerosis patients did not react more frequently to any of the MBP peptides than did healthy control subjects, suggesting that in the majority of cases of multiple sclerosis, autoreactivity to MBP may not play a significant role in the disease process. It remains to be determined whether T-cell responses to these PLP peptides play a pathogenic role in multiple sclerosis.

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